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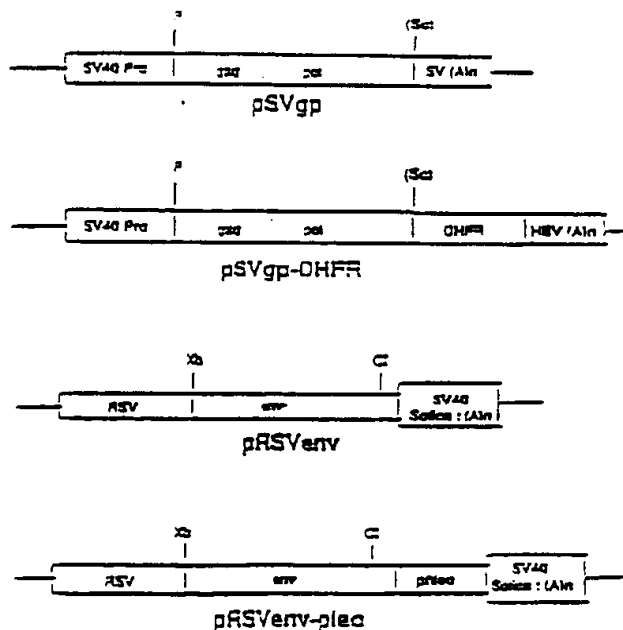
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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**(54) Title:** PACKAGING CELLS**(57) Abstract**

The invention described herein allows the production of recombinant retroviruses (retroviral vector particles) from producer cells which are safer and of higher titre than normal. In addition, methods are provided for making helper cells which, when a recombinant retrovirus genome is introduced to make a producer line, produce particles that are targeted toward particular cell types. Methods are also provided for making recombinant retrovirus systems adapted to infect a particular cell type, such as a tumor, by binding the retrovirus or recombinant retrovirus in the particular cell type. Methods are also provided for producing recombinant retroviruses which integrate in a specific small number of places in the host genome, and for producing recombinant retroviruses from transgenic animals.

## Plasmids Designed to Increase Viral Protein Production



Host cell lines were screened for their ability to efficiently (high titre) rescue a drug resistance retroviral vector (A alpha N2) using replication competent retrovirus to produce the gag/pol and env structural genes ("MA" virus). Titre was measured from confluent monolayers 16 h after a medium change by adding filtered supernatants (0.45 um filters) to  $5 \times 10^4$  NIH 3T3 TK<sup>-</sup> cells on a 6 cm tissue culture plate in the presence of 4 ug/ml polybrene followed by selection in G418.

Data from the screening process is shown in Figure 2. Among the non-murine cell lines which demonstrate the ability to package MoMLV-based vector with high titre are the cell lines CF2, D17, 293, and HT1080. These cell lines were used herein as examples, although any other cells may be tested by such means.

### C. Generation of Packaging Cell

#### (i) gag/pol intermediate

As examples of the generation of gag/pol intermediates for PCL production, D17, 293, and HT1080 were co-transfected with 1 ug of the methotrexate resistance vector, pFR400 (Graham and van der Eb, Virology 52:456-67, 1973), and 10 ug of the MoMLV gag/pol expression vector, pSCV10 (above) by calcium phosphate co-precipitation (D17 and HT1080, see Graham and van der Eb, Virology 52:456-67, 1973), or lipofection (293, see Felgner et al., Proc. Natl. Acad. Sci., USA 84:7413-17, 1987). After selection for transfected cells in the presence of the drugs dipyrimidol and methotrexate, individual drug resistant cell colonies were expanded and analyzed for MoMLV gag/pol expression by extracellular reverse transcriptase (RT) activity (modified from Goff et al., J. Virol. 38:239-48, 1981) and intracellular p30<sup>gag</sup> by western blot using anti p30 antibodies (goat antiserum #77S000087 from the National Cancer Institute). This method identified individual cell clones in each cell type which expressed 10-50x higher levels of both proteins

compared with that of a standard mouse amphotropic PCL, PA317 (Figure 1D and Table 1).

TABLE 1

5 PROPERTIES OF MoMLV GAG/POL-EXPRESSING CELLS

	CELL NAME	RT ACTIVITY (CPM)	p30 <sup>gag</sup> EXPRESSION	LARNL TITRE (CFU/ML)
10	3T3	800	-	N.D.
	PA317	1350	+/-	$1.2 \times 10^3$
	D17	800	-	N.D.
	D17 4-15	5000	+++++	$1.2 \times 10^4$
15	D17 9-20	2000	+++	$6.0 \times 10^3$
	D17 9-9	2200	++	$1.0 \times 10^3$
	D17 9-16	6100	+++++	$1.5 \times 10^4$
	D17 8-7	4000	-	N.D.
	HT1080	900	-	N.D.
20	HTSCV21	16400	+++++	$8.2 \times 10^3$
	HTSCV25	7900	+++	$2.8 \times 10^3$
	HTSCV42	11600	++	$8.0 \times 10^2$
	HTSCV26	4000	-	< 10
	293	600	-	N.D.
25	293 2-3	6500	+++++	$7 \times 10^4$
	293 5-2	7600	+++++	N.D.

The biological activity of these proteins was tested by introducing a retroviral vector, LARNL (see Figure 1B) which expresses both the amphotropic envelope and a Neo<sup>+</sup> marker which confers resistance to the drug, G418. In every case, co-expression of gag/pol in the cell line and env from the vector allowed efficient packaging of the vector as determined by cell-free transfer of G418 resistance to 3T3 cells (titre). Titre was measured from confluent monolayers 16 h after a medium change by adding filtered supernatants (0.45  $\mu$ m filters) to  $5 \times 10^4$  NIH3T3 TK<sup>-</sup> cells on a 6 cm tissue culture plate in the presence of 4  $\mu$ g/ml polybrene followed by selection in G418. Significantly, the vector titres from the cell lines

correlated with the levels of p30<sup>gag</sup> (Table 1). Since the level of env should be the same in each clone and is comparable to the level found in PA317 (data not shown), this indicates that titre was limited by the lower levels of gag/pol in these cells (including PA317). The titre correlated more closely with the levels of p30<sup>gag</sup> than with the levels of RT.

(ii) Conversion of gag/pol lines into amphotropic packaging lines

As examples of the generation of amphotropic PCLs, the gag/pol over-expressors for 293 (termed 2-3) and D17 (termed 4-15) were co-transfected by the same techniques described above except that 1 ug of the phleomycin resistance vector, pUT507 (Mulsant et al., Somat. Cell Mol. Genet. 14:243-52, 1988), and 10 ug of the amphotropic envelope expression vectors, pMLPenvAmSph (for 2-3) or pCMVenvAmNhe (for 4-15) were used. After selection for transfected cells in the presence of phleomycin, individual drug resistant cell colonies were expanded and analyzed for intracellular gp80<sup>env</sup> expression by western blot using anti gp70 (goat antiserum #79S000771 from N.C.I.). Several clones were identified which expressed relatively high levels of both gag/pol and amphi env (PCLs, see Figure 1 for representative data).

In another example of the generation of an amphi PCL, CF2 cells were electroporated (cf. Chu et al., Nucl. Acids Res. 15:1311-26, 1987) with 2 ug of the phleomycin resistance marker, pUT507, 10 ug of pSCV10 (above), and 10 ug of pCMVenvAmNhe (above). After selection for transfected cells in the presence of phleomycin, individual drug resistant cell colonies were expanded and analyzed for intracellular expression of MLV p30<sup>gag</sup> and gp80<sup>env</sup> proteins by western blot using specific antisera. A clone was identified which expressed relatively high levels of both gag/pol and amphi env (Figure 1E).

(iii) Performance of amphotropic packaging cell lines.

A number of these amphi PCLs were tested for their capacity to package retroviral vectors by measuring titre after the introduction of retroviral vectors (Table 2). The measurements were performed using uncloned PCLs, so that the average performance of the lines was calculated.

TABLE 2  
VECTOR TITRE AND HELPER VIRUS  
GENERATION IN AMPHOTROPIC PCLs

CELL TYPE	VECTOR TITRE <sup>a</sup> (+/-HELPER VIRUS <sup>b</sup> )		
	b-Gal	KT-1	N2
PA317	$3.5 \times 10^2$ (N.D.)	$1.0 \times 10^4$ (N.D.)	$3.0 \times 10^5$ (+) <sup>c</sup>
CA	$5.0 \times 10^4$ (N.D.)	$3.0 \times 10^5$ (-) <sup>d</sup>	$2.0 \times 10^6$ (-) <sup>d</sup>
2A	$4.0 \times 10^4$ (N.D.)	$2.0 \times 10^5$ (-) <sup>e</sup>	N.D.
DA	N.D.	N.D.	$2.0 \times 10^5$ (-) <sup>d</sup>
DA2	N.D.	$3.9 \times 10^5$ (-) <sup>d</sup>	N.D.

<sup>a</sup>cfu/ml

<sup>b</sup>as judged by marker rescue assay with MA virus as positive control

<sup>c</sup>after 20 days in culture

<sup>d</sup>after 60 days in culture

<sup>e</sup>after 90 days in culture

Highest titres are obtained when retroviral vectors were introduced into PCLs by infection (Miller et al., Somat. Cell Mol. Genet. 12:175-83, 1986).

However, although amphotropic MLV vectors are known to infect these host cell types, the PCLs are blocked for infection by amphi vector since they express amphi env ("viral interference"). To overcome this problem, vectors containing other viral envelopes (such as xenotropic env or VSV G protein, which bind to cell receptors other than the amphi receptor) were generated in the following manner. Ten ug of the vector DNA of interest was co-

transfected with 10 ug of DNA which expresses either xeno env (pCMVxeno, above) or a VSV G protein expression vector, MLP G, onto a cell line which expresses high levels of MoMLV gag/pol such as 2-3 cell (see above). The resultant vector containing xenotropic env or VSV G protein, respectively, was produced transiently in the co-transfected cells and after 2 days cell free supernatants were added to the potential PCLs, and vector-infected cells were identified by selection in G418. Both types of vector efficiently infected the amphi-blocked cells and after G418 selection cell free supernatants were collected from the confluent monolayers and titred on NIH 3T3 TK<sup>-</sup> cells as described above. The cell clones with the highest titre were chosen as PCLs and referred to as DA (D17 amphi), 2A (293 amphi), and CA (CF2 amphi), respectively. In no case was helper virus detected in the currently described PCLs, even when a retroviral vector (N2) which has a high probability of generating helper virus (Armentano et al., J. Virol. 62:1647-50, 1987) was introduced into the PCLs and the cells passaged for as long as 2 months (3 months for vector KT-3). On the other hand, the same vector introduced into the PA317 cell line generated helper virus within 3 weeks of continual passaging.

(iv) Conversion of gag/pol lines into xenotropic packaging cell lines.

As examples of the generation of xenotropic PCLs, the gag/pol over-expressors for D17 (4-15) and HT1080 (SCV21) were co-transfected by the same techniques described above except that 1 ug of either the phleomycin resistance vector, pUT507 (for SCV21), or the hygromycin B resistance marker, pY3 (for 4-15, see Blochlinger and Diggelmann, Mol. Cell Biol. 4:2929-31, 1984), and 10 ug of the xenotropic envelope expression vector, pCMVxeno (above) was used. After selection for transfected cells in the presence of phleomycin or hygromycin, respectively, individual drug resistant cell colonies were expanded and

analyzed for intracellular expression of MLV p30<sup>gag</sup> and gp75<sup>env</sup> proteins by western blot using specific antisera. Clones were identified which expressed relatively high levels of both gag/pol and xeno env (Figure 1F).

5 (v) Performance of xenotropic packaging cell lines.

A number of these potential xeno PCLs were tested for their capacity to package retroviral vectors by measuring titre after the introduction of retroviral  
10 vectors (Table 3).

TABLE 3  
VECTOR TITRE ON XENOTROPIC PCLs

15	CELL CLONE		KT-1 TITRE (CFU/ML) ON HT1080 CELLS
	HT1080	SCV21	$1.0 \times 10^5$
		XF1	$1.0 \times 10^5$
20		XF7	$1.0 \times 10^5$
		XF12 (HX)	$4.5 \times 10^5$
	D17	4-15	
		X6	$9.0 \times 10^4$
25		X10 (DX)	$1.3 \times 10^5$
		X23	$8.0 \times 10^4$

As described above, vector containing VSV G  
30 protein was produced transiently in 2-3 cells. After 2 days, cell free supernatants were added to the xeno PCLs and after G418 selection cell free supernatants were collected from the confluent monolayers and titred as described above except that HT1080 cells, which are  
35 infectable by xeno vector, was used instead of NIH 3T3 TK<sup>-</sup> cells which are resistant to xeno vector. The cell clones with the highest titre were chosen as PCLs and referred to as DX (D17 xeno) and HX (HT1080 xeno), respectively.

The propensity of the PCLs described above to generate helper virus was tested even more stringently by co-cultivating amphi and xeno PCLs containing the vector, N2. Since amphi vector can infect the xeno PCLs and vice versa, this allows continuous cross-infection events, each of which increases the probability of generating helper virus. As an example, 2A cells containing N2 were co-cultivated with HX cells containing N2. After 23 days, the cultures were still free of amphi and xeno viruses as judged by a vector rescue assay on 293 or Mus dunni cells, both of which can detect amphi and xeno viruses (Table 4).

TABLE 4  
HIGH STRINGENCY ANALYSIS FOR PCL TENDENCY TO GENERATE  
HELPER VIRUS

15

TEST MATERIAL	HELPER VIRUS ASSAY
AMPHOTROPIC VIRUS	+
20 XENOTROPIC VIRUS	+
PA317 + N2 (21d)	+
2A + HX + N2 (23d)	-

25 (vi) Conversion of gag/pol lines into polytropic packaging cell lines.

As an example of the generation of a polytropic PCL, the gag/pol over-expressor for HT1080 (SCV21) was co-transfected by the same techniques described above, except that 1 ug of the phleomycin resistance vector, pUT507, and 10 ug of the polytropic envelope expression vector, pCMVMCF (above) was used. After selection for transfected cells in the presence of phleomycin, individual drug resistant cell colonies were expanded and analyzed for intracellular expression of MLV gp70<sup>env</sup> protein by western blot using specific antiserum. Clones were identified

which expressed relatively high levels of both gag/pol (not shown) and polytropic env (Figure 1G).

(vii) Performance of polytropic packaging cell lines.

5 One of these potential poly PCLs (clone 3) was tested for the capacity to package retroviral vectors by measuring titre after the introduction of retroviral vectors (Table 5).

10

TABLE 5  
HOST-RANGE OF POLYTROPIC VECTOR FROM HP CELLS

	CELL LINE	SPECIES	b-Gal TITRE
15	3T3	MURINE	$1.0 \times 10^4$
	PA317	MURINE	$1.0 \times 10^4$
	Mv-1-Lu	MINK	$5.0 \times 10^3$
	FRh1	MACAQUE	< 10
	HT1080	HUMAN	< 10
20	HeLa	HUMAN	< 10
	WI 38	HUMAN	< 10
	DETROIT 557	HUMAN	< 10
	SUP TI	HUMAN	< 10
	CEM	HUMAN	< 10
25	U937	HUMAN	< 10
	293	HUMAN	$2.0 \times 10^4$

This cell clone was chosen as PCL and referred to as HP (HT1080 poly). As described above, vector  
 30 containing VSV G protein was produced transiently in 2-3 cells and after 26 days, cell free supernatants were added to the polytropic PCL (HP). After G418 selection, cell free supernatants were collected from the confluent monolayers and titred as described above on a variety of  
 35 cell lines. The infection of human cells was very restricted, with all cell lines tested being negative with the exception of 293 cells.

Although the factors that lead to efficient infection of specific cell types by retroviral vectors are not completely understood, it is clear that because of their relatively high mutation rate, retroviruses may be adapted for markedly improved growth in cell types in which initial growth is poor, simply by continual reinfection and growth of the virus in that cell type (the adapter cell). This can also be achieved using viral vectors that encode some viral functions (e.g., env), and which are passed continuously in cells of a particular type which have been engineered to have the functions necessary to complement those of the vector to give out infectious vector particles (e.g., gag/pol). For example, one can adapt the murine amphotropic virus 4070A to human T-cells or monocytes by continuous growth and reinfection of either primary cell cultures or permanent cell lines such as Sup T1 (T-cells) or U937 (monocytes). Once maximal growth has been achieved, as measured by reverse transcriptase levels or other assays of virus production, the virus is cloned out by any of a number of standard methods, the clone is checked for activity (i.e., the ability to give the same maximal growth characteristic on transfection into the adapter cell type) and this genome used to make defective helper genomes and/or vectors which in turn, in an appropriately manufactured helper or producer line, will lead to production of viral vector particles which infect and express in the adapter cell type with high efficiency ( $10^7$  -  $10^9$  infectious units/ml).

#### VII. Alternative Viral Vector Packaging Techniques

Two additional alternative systems can be used to produce recombinant retroviruses carrying the vector construct. Each of these systems takes advantage of the fact that the insect virus, baculovirus, and the mammalian viruses, vaccinia and adenovirus, have been adapted recently to make large amounts of any given protein for which the gene has been cloned. For example, see Smith

et al. (Mol. Cell. Biol. 3:12, 1983); Piccini et al. (Meth. Enzymology, 153:545, 1987); and Mansour et al. (Proc. Natl. Acad. Sci. USA 82:1359, 1985).

These viral vectors can be used to produce  
5 proteins in tissue culture cells by insertion of appropriate genes into the viral vector and, hence, could be adapted to make retroviral vector particles.

Adenovirus vectors are derived from nuclear replicating viruses and can be defective. Genes can be  
10 inserted into vectors and used to express proteins in mammalian cells either by in vitro construction (Ballay et al., EMBO J. 4:3861, 1985) or by recombination in cells (Thummel et al., J. Mol. Appl. Genetics 1:435, 1982).

One preferred method is to construct plasmids  
15 using the adenovirus Major Late Promoter (MLP) driving: (1) gag/pol, (2) env, (3) a modified viral vector construct. A modified viral vector construct is possible because the U3 region of the 5' LTR, which contains the viral vector promoter, can be replaced by other promoter  
20 sequences (see, for example, Hartman, Nucl. Acids Res. 16:9345, 1988). This portion will be replaced after one round of reverse transcriptase by the U3 from the 3' LTR.

These plasmids can then be used to make adenovirus genomes in vitro (Ballay et al., op. cit.), and  
25 these transfected in 293 cells (a human cell line making adenovirus E1A protein), for which the adenoviral vectors are defective, to yield pure stocks of gag/pol, env and retroviral vector carried separately in defective adenovirus vectors. Since the titres of such vectors are  
30 typically  $10^7$ - $10^{11}$ /ml, these stocks can be used to infect tissue culture cells simultaneously at high multiplicity. The cells will then be programmed to produce retroviral proteins and retroviral vector genomes at high levels. Since the adenovirus vectors are defective, no large  
35 amounts of direct cell lysis will occur and retroviral vectors can be harvested from the cell supernatants.

Other viral vectors such as those derived from unrelated retroviral vectors (e.g., RSV, MMTV or HIV) can be used in the same manner to generate vectors from primary cells. In one embodiment, these adenoviral  
5 vectors are used in conjunction with primary cells, giving rise to retroviral vector preparations from primary cells.

In some cases, gene products from other viruses may be used to improve the properties of retroviral packaging systems. For instance, HIV rev protein might be  
10 included to prevent splicing of HIV env or HIV gag/pol MLV vectors or HIV sor might increase the infectivity of T cells by free virus as it does with HIV (See Fischer et al., Science 237:888-893, 1987).

In an alternative system (which is more truly  
15 extracellular), the following components are used:

1. gag/pol and env proteins made in the baculovirus system in a similar manner as described in Smith et al. (supra) (or in other protein production systems, such as yeast or E. coli);
- 20 2. viral vector RNA made in the known T7 or SP6 or other in vitro RNA-generating system (see, for example, Flamant and Sorge, J. Virol. 62:1827, 1988);
3. tRNA made as in (2) or purified from yeast or mammalian tissue culture cells;
- 25 4. liposomes (with embedded env protein); and
5. cell extract or purified necessary components (when identified) (typically from mouse cells) to provide env processing, and any or other necessary cell-derived functions.

30 Within this procedure (1), (2) and (3) are mixed, and then env protein, cell extract and pre-liposome mix (lipid in a suitable solvent) added. It may, however, be necessary to earlier embed the env protein in the liposomes prior to adding the resulting liposome-embedded  
35 env to the mixture of (1), (2), and (3). The mix is treated (e.g., by sonication, temperature manipulation, or rotary dialysis) to allow encapsidation of the nascent

viral particles with lipid plus embedded env protein in a manner similar to that for liposome encapsidation of pharmaceuticals, as described in Gould-Fogerite et al., Anal. Biochem. 148:15, 1985). This procedure allows the  
5 production of high titres of replication incompetent recombinant retroviruses without contamination with pathogenic retroviruses or replication-competent retroviruses.

10 VIII. Cell Line-Specific Retroviruses - "Hybrid Envelope"

The host cell range specificity of a retrovirus is determined in part by the env gene products. For example, Coffin, J. (RNA Tumor Viruses 2:25-27, Cold Spring Harbor, 1985) notes that the extracellular  
15 component of the proteins from murine leukemia virus (MLV) and Rous Sarcoma virus (RSV) are responsible for specific receptor binding. The cytoplasmic domain of envelope proteins, on the other hand, are understood to play a role in virion formation. While pseudotyping (i.e., the  
20 encapsidation of viral RNA from one species by viral proteins of another species) does occur at a low frequency, the envelope protein has some specificity for virion formation of a given retrovirus. The present invention recognizes that by creating a hybrid env gene  
25 product (i.e., specifically, an env protein having cytoplasmic regions and exogenous binding regions which are not in the same protein molecule in nature) the host range specificity may be changed independently from the cytoplasmic function. Thus, recombinant retroviruses can  
30 be produced which will specifically bind to preselected target cells.

In order to make a hybrid protein in which the receptor binding component and the cytoplasmic component are from different retroviruses, a preferred location for  
35 recombination is within the membrane-spanning region of the cytoplasmic component. Example 10 describes the construction of a hybrid env gene which expresses a

protein with the CD4 binding portion of the HIV envelope protein coupled to the cytoplasmic domain of the MLV envelope protein.

5

### EXAMPLE 3

#### Hybrid HIV-MLV Envelopes

A hybrid envelope gene is prepared using in vitro mutagenesis (Kunkel, Proc. Natl. Acad. Sci. USA 82:488-492, 1985) to introduce a new restriction site at an appropriate point of junction. Alternatively, if the two envelope sequences are on the same plasmid, they can be joined directly at any desired point using in vitro mutagenesis. The end result in either case is a hybrid gene containing the 5' end of the HIV gp 160 and the 3' end of MLV p15E. The hybrid protein expressed by the resulting recombinant gene is illustrated in Figure 2 and contains the HIV gp120 (CD4 receptor binding protein), the extracellular portion of HIV gp 41 (the gp 120 binding and fusogenic regions), and the cytoplasmic portion of MLV p15E, with the joint occurring at any of several points within the host membrane. A hybrid with a fusion joint at the cytoplasmic surface (joint C in Figure 2) causes syncytia when expressed in Sup T1 cells. The number of apparent syncytia are approximately one-fifth that of the nonhybrid HIV envelope gene in the same expression vector. Syncytia with the hybrid occurs only when the rev protein is co-expressed in trans. A hybrid with a fusion joint at the extracellular surface (joint A in Figure 2) gives no syncytia while hybrid B (in the middle of the transmembrane regions) gives approximately five-fold less syncytium on Sup T1 cells than hybrid C.

While Example 3 illustrates one hybrid protein produced from two different retroviruses, the possibilities are not limited to retroviruses or other viruses. For example, the receptor binding portion of human interleukin-2 may be combined with the envelope protein of MLV to target vectors to cells with IL-2 receptors. In this

case, a recombination would preferably be located in the gp 70 portion of the MLV env gene, leaving an intact p15E protein. Furthermore, the foregoing technique may be used to create a recombinant retrovirus with an envelope protein which recognizes antibody Fc segments. Monoclonal antibodies which recognize only preselected target cells only could then be bound to such a recombinant retrovirus exhibiting such envelope proteins so that the retrovirus would bind to and infect only those preselected target cells. Alternatively, a hybrid envelope with the binding domain of avidin would be useful for targeting cells' "images" in a patient or animal with biotinylated antibodies or other ligands. The patient would first be flooded with antibodies, and then antibody binding nonspecifically allowed to clear from the patient's system, before administering the vector. The high affinity ( $10^{-15}$ ) of the avidin binding site for biotin would then allow accurate and efficient targeting to the original tissue identified by the monoclonal "image."

The approach may also be used to achieve tumor-specific targeting and killing by taking advantage of three levels of retroviral vector specificity; namely, cell entry, gene expression, and choice of protein expressed. Retroviral vectors enter cells and exert their effects at intracellular sites. In this respect their action is quite unique. Using this property, and the three levels of natural retroviral specificity (above), retroviral vectors may be engineered to target and kill tumor cells.

The overall goal of targeting of retrovirus to tumor cells may be accomplished by two major experimental routes; namely, a) selection in tissue culture (or in animals) for retroviruses that grow preferentially in tumor cells; or b) construction of retroviral vectors with tissue (tumor) -specific promoters with improvements being made by in vitro passage, and negative and positive drug-sensitivity selection.

Vectors suitable for selectively infecting selected cell types, such as a tumor cell, may generally be prepared by (a) continuously passaging a virus in cells of the selected cell type until the virus has genetically mutated and a predominant fast growing strain has evolved; 5 (b) isolating the mutated and fast growing strain; (c) identifying and isolating the components of the mutated strain responsible for the preferential growth of the mutated virus; (d) inserting the identified and isolated 10 components as substitutes for counterpart components in a producer cell based upon the virus (prior to continuous passage); and (e) culturing the producer cell to produce the vector.

At least four selective protocols may be 15 utilized to select for retrovirus which grow preferentially in tumor cells; namely, 1) "Env Selection by Passage In Vitro," wherein selection of retrovirus with improved replicative growth ability is accomplished by repeated passage in tumor cells; 2) "Selection with a Drug 20 Resistance Gene," wherein genetic selection for tumor "specific" retroviruses is based on viral constructs containing a linked drug resistance gene; 3) "Hybrid-Env," wherein selection (by protocol #1 or #2, above) of retrovirus with tumor-"specificity" is initiated from a 25 construct containing a hybrid envelope gene which is a fusion of a tumor receptor gene (i.e., an anti-tumor antibody H-chain V-region gene fused with env; or, a growth receptor fused with env); in this case selection begins at a favorable starting point, e.g., an env which 30 has some specificity for tumor cells; or 4) "Selection by Passage In Vitro and Counter Selection by Co-cultivation with Normal Cells," wherein growth in tumor cells is selected-for by repeated passage in mixtures of drug-resistant tumor cells and drug-sensitive normal cells.

35 With respect to retroviral vector constructs carrying tissue (tumor) -specific promoters, biochemical markers with different levels of tissue-specificity are

well known, and genetic control through tissue-specific promoters is understood in some systems. There are a number of genes whose transcriptional promoter elements are relatively active in rapidly growing cells (i.e., transferring receptor, thymidine kinase, etc.) and others whose promoter/enhancer elements are tissue specific (i.e., HBV enhancer for liver, PSA promoter for prostate). Retroviral vectors and tissue-specific promoters (present either as an internal promoter or within the LTR) which can drive the expression of selectable markers and cell cycle genes (i.e., drug sensitivity, Eco gpt; or HSVtk in TK-cells). Expression of these genes can be selected for in media containing mycophenolic acid or HAT, respectively. In this manner, tumor cells containing integrated provirus which actively expresses the drug resistance gene will survive. Selection in this system may involve selection for both tissue-specific promoters and viral LTRs. Alternatively, specific expression in tumor cells, and not in normal cells, can be counter-selected by periodically passaging virus onto normal cells, and selecting against virus that express Eco gpt or HSVtk (drug sensitivity) in those cells (by thioxanthine or acyclovir). Infected cells containing integrated provirus which express Eco gpt or tk phenotype will die and thus virus in that cell type will be selected against.

#### IX. Site-Specific Integration

Targeting a retroviral vector to a predetermined locus on a chromosome increases the benefits of gene-delivery systems. A measure of safety is gained by direct integration to a "safe" spot on a chromosome, i.e., one that is proven to have no deleterious effects from the insertion of a vector. Another potential benefit is the ability to direct a gene to an "open" region of a chromosome, where its expression would be maximized. Two techniques for integrating retroviruses at specific sites are described below.